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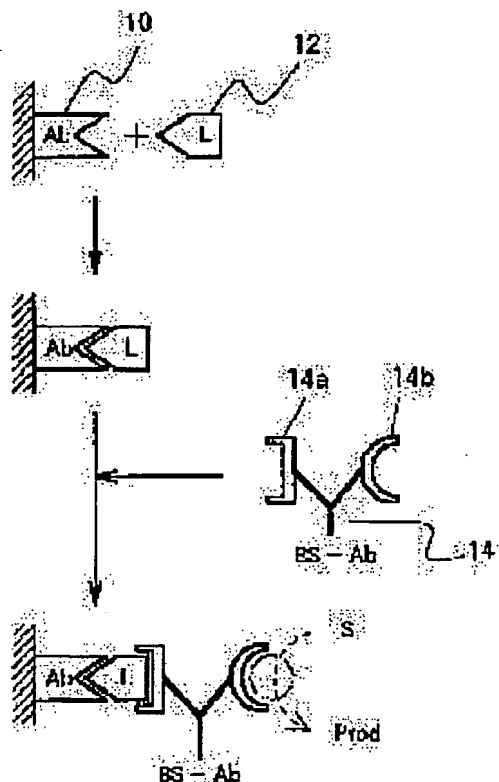
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(54) ANTIBODY ENZYME IMMUNOASSAY

(57)Abstract:

PURPOSE: To enable analysis with high sensitivity and high reproducibility without causing deterioration of S/N by bringing a two-head antibody into contact with a ligand, and measuring the activity of the antibody enzyme of the two-head antibody being coupled to the ligand, the two-head antibody consisting of a moiety of an antibody for the ligand and a moiety of the antibody enzyme which can generate signals.

CONSTITUTION: Antigens (ligand L) 12 in a sample are coupled to a solid-phase antibody 10 (first immunoreaction). Those antigens which are not yet coupled are removed (washing). A two-head antibody(Bs-Ab) comprising a moiety 14a of



the ligand specific antibody and a moiety 14b of an enzyme antibody is added and coupled to the antigens 12 being coupled to the solid-phase antibody 10 (second immunoreaction). Those two-head antibodies 14 which are not yet coupled to the antigens 12 are removed (washing). An enzyme substrate (S) is added to detect a product (Prod) so as to measure the amount of the coupled two-head antibodies (enzyme reaction).

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CLAIMS

[Claim(s)]

[Claim 1] The antibody enzyme immunity analysis method characterized by measuring the activity of the antibody enzyme of the two-animal antibody which the two-animal antibody which consists of a moiety object of an antibody over ligand and a moiety object of the antibody enzyme which can generate a detectable signal was contacted to ligand, and was combined with ligand.

[Claim 2] The antibody enzyme immunity analysis method characterized by measuring the antibody enzyme activity of the two-animal antibody which the two-animal antibody which consists of a moiety object of an antibody over ligand and a moiety object of the antibody enzyme which can generate a detectable signal is contacted to ligand, and has not been combined with ligand.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[Industrial Application] This invention relates to the antibody enzyme immunity analysis method using a two-animal antibody in detail about the immunity analysis method which uses an antibody enzyme.

[0002]

[Background of the Invention] Analysis of blood, urine, etc. is dramatically useful to a diagnosis of symptoms, or the judgment of therapy progress, and has played the important role in the field of the clinical laboratory test. The approach of measuring immunologically as analytical method of such a minor constituent (ligand) using the antibody to this minor constituent is applied widely (for example, an "immunology illustration lei TEDDO" Tomio Tada translation, Nankodo, 1990, p327 -339 reference). An immunoreaction can carry out [analyze / the equilibrium of an antibody and an antigen / are the reaction of high compatibility and / specific and / between an antigen and an antibody, / using a labelled antigen or a labelled antibody] the quantum of the antigen of the measuring object. Enzyme immunoassay (enzyme immunoassay; EIA) used the chemistry magnification by the enzyme as this indicator using the enzyme. It is briskly used as analytical method [that enzyme immunoassay is simple and high sensitivity] in recent years, and the detail is indicated by for example, Eiji Ishikawa, crossing Akio edits, and "enzyme immunoassay" (KYORITSU SHUPPAN, 1987).

[0003] The enzyme immunoassay most often because of current and antigen measurement used is the so-called sandwich technique. A sandwich technique is a measuring method of a polyvalent antigen with two or more antigen decision parts, and two kinds of antibodies to a different antigen decision part are used for it. The typical sandwiches EIA method can be performed, for example at the following steps.

1. Combine the antigen in a sample with a solid phase-ized antibody (the 1st

antibody) (the 1st immunoreaction).

2. Remove an uncombined antigen (washing).

3. Add an enzyme labelled antibody (the 2nd different antibody from a solid phase-ized antibody), and make it combine with the antigen on solid phase (the 2nd immunoreaction).

4. Remove an uncombined enzyme labelled antibody (washing).

5. Measure the amount of enzyme labelled antibodies which added the enzyme substrate and has been combined (enzyme reaction).

From such an approach, the indicator of the 2nd antibody needed to be carried out with the enzyme, the enzyme labelled antibody needed to be made, and the antibody and the enzyme were mainly chemically combined by it using the crosslinking reagent (for example, Eiji Ishikawa, the Kawai **** Miyai **** "enzyme immunoassay" (Igaku-Shoin, 1987)).

[0004] In enzyme immunoassay, the engine performance of the enzyme labelled antibody to be used has big effect on sensitometry, repeatability, etc. However, the enzyme labelled antibody which is made to carry out the chemical bond of an antibody and the enzyme, and is obtained has a problem in respect of this engine performance.

[0005] Although glutaraldehyde typical as a crosslinking reagent joins together and constructs a bridge with an antibody and the amino group of an enzyme, the association is random, reacts to an antibody and the amino group of an enzyme indiscriminately, and spoils antibody activity and enzyme activity. Moreover, indicator effectiveness is low, in order that only an enzyme may carry out a polymerization or only an antibody may carry out a polymerization. Moreover, the homopolymer and the heteropolymer increased as a result of the random reaction, and since separation with the enzyme labelled antibody (enzyme: the complex combined at a rate of antibody =1:1 is ideal) to need was difficult for them once these polymers are formed, they had become the cause of reducing the sensibility and the repeatability of the EIA method.

[0006] A periodic acid method oxidizes the sugar chain of an enzyme, makes an aldehyde group form, and reacts and combines this aldehyde group and the amino group of an antibody. By this approach, although there is little lowering of enzyme activity, since there is no selectivity over the amino group of an antibody, lowering of antibody activity is not escaped. Moreover, it is inapplicable to an enzyme without a sugar chain.

[0007] The maleimide method makes the maleimide radical introduced into the enzyme at the thiol group in the hinge region of an IgG antibody join together and construct a bridge. Since an enzyme is selectively combinable with a part for the hinge region which is distant from an antibody active site by this approach, there is little lowering of antibody activity. However, since there is no selectivity over the amino group of an enzyme, if there are two or

more amino groups in an enzyme, as for enzyme activity, some will fall. Moreover, if the antibody more than dyad will combine with the enzyme of one molecule, the concentration of the marker enzyme which receives per antibody 1 molecule becomes low, and lifting of sensibility cannot be desired. [0008] Although the enzyme-labeling method by the chemical bond is in others variously, each enzyme labelled antibody obtained has the fault with the much the same above. Moreover, the enzyme labelled antibody by these chemical bonds also tends to produce the defect in which nonspecific adsorbent [over the solid phase of a microtiter plate, a bead, etc.] increases, in order for molecules with large molecular weight called an antibody and an enzyme to join together. Therefore, there was also a problem that the sensibility as expected was no longer obtained, or repeatability became scarce.

[0009] Moreover, we are two-animal antibodies (BS antibody; Bi-specific antibody) about an enzyme instead of using enzyme-labelled antibody complex like before combined chemically. The approach of making it joining together immunologically and performing enzyme immunity analysis was also developed, and this engine performance was also examined (Japanese Patent Application No. 3-113774). Improvement remarkable in sensibility and repeatability was found by the approach using this two-animal antibody. However, the new problem that a S/N ratio falls by the nonspecific adsorption of an additive enzyme has produced this approach in order to have to add a lot of enzymes to the system of reaction.

[0010]

[Objects of the Invention] This invention is made in view of the above situations, there is little lowering of antibody activity and good analysis of high sensitivity and repeatability aims at offering the immunoassay which can be performed without being accompanied by lowering of a S/N ratio.

[0011]

[Elements of the Invention] The object of such this invention contacted to ligand the two-animal antibody which consists of a moiety object of an antibody over ligand, and a moiety object of the antibody enzyme which can generate a detectable signal, and was attained by the antibody enzyme immunity analysis method characterized by measuring the activity of the antibody enzyme of the two (or it has not carried out) animal antibody combined with ligand.

[0012]

[Function] It is the two-animal antibody (BS antibody; Bi-specific antibody) of an antibody enzyme instead of using enzyme-labelled antibody complex like before combined chemically in this invention. It uses and immunity analysis is performed. This is based on the knowledge by the artificer that lowering of the sensibility which poses a problem is no longer seen, by using

the two-animal antibody which consists of a moiety object of the antibody enzyme which can generate a detectable signal, and a moiety object of the specific antibody which has the antigenic specificity over the specimen (ligand).

[0013] A two-animal antibody is the same dimer structure as the usual antibody, and since chemical qualification is not carried out, antibody activity is not spoiled. Moreover, the enzyme activity of an antibody enzyme is not spoiled, either. Moreover, since addition of an enzyme etc. is not needed other than this two-animal antibody, lowering of the S/N ratio accompanying the non-specific adsorption of an additive enzyme etc. is not produced.

[0014]

[Detailed explanation of the configuration of invention]

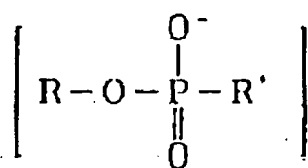
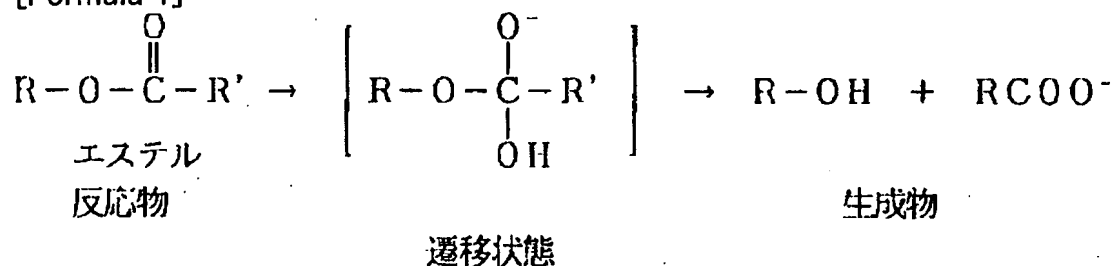
the antibody which has the capacity to change the specific binding pair to the antibody enzyme in antibody enzyme this invention at another product -- saying . In other words, it is the thing holding enzyme activity (or catalytic activity) of an antibody. Antibody enzyme (Antibody Enzyme) Moreover, there is also a thing to which it is referred to as a catalytic antibody (catalytic Antibody), Abzyme, etc. and which is called.

[0015] Generally, an antibody is characterized as protein which identifies a specific molecule (antigen) and is combined specifically. On the other hand, it not only combines with a specific molecule (enzyme substrate) specifically the enzyme which is the same protein, but it has the function which carries out the catalyst of the chemical reaction of the molecule. That is, although an enzyme and an antibody are the specific matter and the protein in the living body which can build a joint pair, an antibody is distinguishable from an enzyme in that there is no capacity which carries out the catalyst of the chemical reaction. However, the attempt whether it could acquire the property which an antibody and an enzyme share, i.e., an antibody which carries out the catalyst of the chemical reaction on the basis of the character in which it joins together specifically, since an antibody can obtain a specific thing to almost all a principle target's molecules was made. By early research, since it was what produces the antibody to a substrate, it did not simply come to find an antibody with the catalyst effectiveness (Biochemistry, 5, 2836 (1966), FEBS Letter, 100, and 137 (1979)). On the other hand, P.G.Schulz and others, R.A.Lerner and others are L.Pauling "the active center of an enzyme has the transition state of a reaction, and complementary structure". Based on the idea, the antibody to the analog of the transition state of a reaction was produced, and it discovered that this antibody had catalytic activity (Science, 234, 1570 (1986), Science, 234, 1566 (1986)). The antibody which has such catalytic activity by many researchers is produced, and the idea of an antibody enzyme (a catalytic antibody, Abzyme) has come [then,] to be accepted widely.

[0016] A transition state analog is the stable analog which the matter (intermediate field) and form which are in a transition state in a certain enzyme reaction, and the charge bore a strong resemblance to, and such a transition state analog works as an antigen, and it combines with the matter in the transition state of a reaction process, and the antibody which guided the antibody and was able to do it stabilizes this, and functions as a catalyst further. For example, at the hydrolysis reaction of carboxylate, the ester which is a reactant takes the molecular structure on the flat surface which generally does not have a charge. Hydrolysis starts by the attack of a water molecule, and after it passes through tetrahedral intermediate (transition state) with a charge, it follows progress of decomposing into a carboxylic acid and alcohol promptly. The joint direction between atoms changes in this intermediate field, and the interatomic distance is also about 1.2. It is extended to like twice. This cannot be isolated from such an unstable description and the antibody cannot be obtained (following-ized 1 reference).

[0017]

[Formula 1]



リン酸エステル
遷移状態アナログ

[0018] However, if the carbon atom (C) of the center of this tetrahedral structure is transposed to the Lynn atom (P), it will become the stable compound called the phosphoric ester which takes the well alike configuration. And the bond distance between Lynn-oxygen is longer than association between usual carbon-oxygen about 20%, and close to the bond distance of a actual transition state. It is known that phosphoric ester equipped with such [actually] a property will check a certain kind of hydrolase. By carrying out immunity, using such a transition state analog as

an antigen, an antibody with this enzyme activity, i.e., an antibody enzyme, can be obtained.

[0019] The antibody enzyme used as one side of the moiety object of the two-animal antibody in this invention means the antibody which has such enzyme activity. In this invention, an antibody enzyme is used as a substitute of conventional marker enzyme. Therefore, the antibody enzyme used here can produce the product or decomposition product which can generate a detectable signal from a substrate. For example, there are some which make a substrate matter [as / whose product produced by the esterolysis in said formula 1 is coloring matter]. In addition, it is desirable to choose what produces a monoclonal antibody and has [rather than] high catalytic activity using the polyclonal antibody obtained as an antibody enzyme by carrying out immunity of the transition state analog as it is as it is, and to make this into the ingredient of the two-animal antibody of this invention.

[0020] Although a two-animal antibody immunoglobulin (antibody) is classified into some classes not from one kind of thing but from the chemical structure, the basic structure is structure looked at by IgG. That is, molecular-weight about 25,000 L chain, and molecular-weight about 50,000 H chain carry out an S-S bond (disulfide bond), and it becomes one unit (moiety object), and these equivalent moiety object dimer-izes by the S-S bond of an H chain further, and forms one IgG molecule. An antigen binding site (Fab part) exists in each moiety object, respectively. That is, IgG has the capacity (divalent [of the same kind]) combined with two antigen molecules, although antigenic specificity is one. The concept itself which is going to make the antibody (the so-called different-species divalent two-animal antibody) which has two different antigenic specificity by processing chemically this divalent IgG of the same kind already exists, and various approaches are developed. The two-animal antibody used by this invention can be made with these well-known techniques, and consists of a moiety object of the antibody enzyme which can generate a detectable signal, and a moiety object of the specific antibody to the specimen (ligand).

[0021] For example, after carrying out pepsin digestion of two sorts of IgG and removing Fc part according to the Nisonoff approaches, it returns, respectively, and it is Fab' of a moiety object. It obtains and a two-animal antibody can be created by reoxidating this (Arch.Biochem.Biophys., 90, and 460-462 (1961)). Moiety object Fab' of the antigenic specificity same besides the different-species bifunctional antibody which moiety object Fab' of the antibody which is different from each other combined by this approach The united (self-association) bifunctional antibody of the same kind is also formed. Therefore, a different-species bifunctional antibody is separated and refined from a bifunctional antibody of the same kind in this case. or Brennan
** -- an approach -- following -- moiety object Fab' A sulfhydryl group may

be temporarily protected for one side with a nitro benzoic acid (Science, 229, and 81 (1985)). Thereby, a different-species divalent two-animal antibody can be selectively created from two sorts of monoclonal antibodies. The approach of Okumura and others who facilitated the technique of Brennan and others further may be followed (JP,2-76899,A).

[0022] In addition, although F(ab')₂ which all removed Fc part is used in the above-mentioned two-animal antibody, it may return without removing Fc part, and a moiety object may be acquired, and this may be oxidized and combined with other moiety objects. However, it is easy to carry out nonspecific adsorption of the Fc part to solid phase. Therefore, Fab' obtained by carrying out pepsin digestion of IgG, removing Fc part for high sensitivity measurement, and returning further Considering as the ingredient of a two-animal antibody is desirable.

[0023] Moreover, a two-animal antibody can unite two kinds of hybridomas, and can produce them also by producing a hetero hybridoma. Moreover, it is producible also by uniting a spleen cell with a hybridoma. The creation approach of a monoclonal antibody, and the purification approach and the acquisition approach of F(ab')₂ fragment can be acquired by the approach indicated by various compendiums (for example, Tomiyama ** 2 volume, "monoclonal antibody experiment manual" *****, 1988).

[0024] The antibody enzyme immunity analysis method by method Homoto invention of analysis can specifically be performed as follows. (Refer to drawing 1).

- 1) Combine the antigen 12 in a sample (ligand L) with the solid phase-ized antibody 10 (the 1st immunoreaction).
- 2) Remove the uncombined antigen 12 (washing).
- 3) Add the two-animal antibody (BS-Ab) 14 which consists of moiety object 14a of a ligand specific antibody, and moiety object 14b of an enzyme-labelled antibody, and make it combine with the antigen 12 combined with the solid phase-ized antigen 10 (the 2nd immunoreaction).
- 4) Remove an uncombined two-animal antibody to an antigen 12 (washing).
- 5) Add an enzyme substrate (S) and measure the amount of two-animal antibodies combined by detecting a product (Prod) (enzyme reaction).

[0025] Although the antibody enzyme activity of the two-animal antibody which the above applied this invention to the sandwich technique, and was combined with solid phase is measured, you may make it measure the activity of the antibody enzyme combined with the two-animal antibody of the isolation which was not combined with solid phase. Moreover, the practice of this invention is not necessarily limited to such a sandwich technique. For example, the solid phase antigen of a constant rate and the antigen in a sample (ligand) are made to compete to the two-animal antibody of a constant rate, and you may make it calculate the amount of the

two-animal antibody combined with solid phase by antibody enzyme activity measurement. Moreover, a two-animal antibody is beforehand combined with a solid phase antigen, and you may make it measure the amount of the two-animal antibody on the solid phase which decreased by contention with the antigen in the sample added later (ligand) (the so-called substitution method).

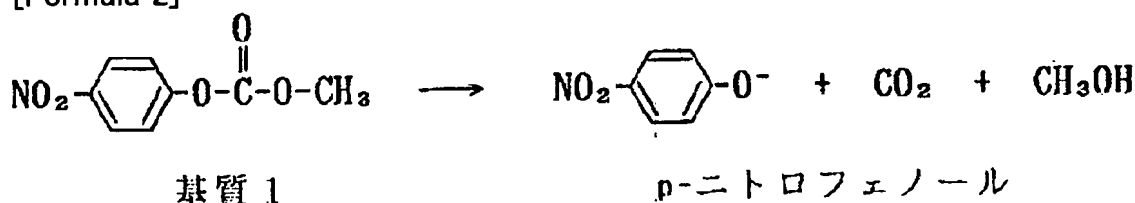
[0026]

[The synthetic example 1]

The enzyme-labelled antibody which carries out the catalyst of this was produced supposing the reaction which generates p-nitrophenol (yellow: absorb to 400nm) from a substrate 1 by the synthetic following reaction formula of the hapten for antibody enzymes.

[0027]

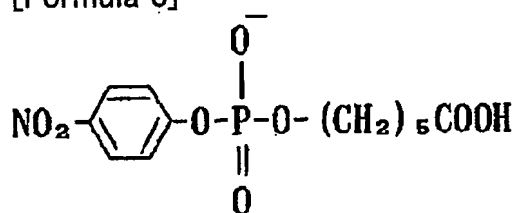
[Formula 2]



[0028] The compound of the following structure expression was first compounded as a transition state analog (hapten) of the above-mentioned reaction.

[0029]

[Formula 3]



[0030] The outline of the reaction synthesis path is shown in drawing 2. First, the compound 1 (17g; 0.1 mol) and compound 2 (20g; 0.1 mol) of drawing 2 were mixed, and it heated in 160 ** for 7 hours. After cooling, when the low-boiling point part was distilled out by distillation, the target compound 3 was obtained as a 13g colorlessness oil. A compound 3 (6.5g; 0.026 mol) is dissolved in the ethanol of 10mL(s), and it is 12 Ns. The hydrochloric acid was added 80 mLs. It is mixture to 160 ** 2.5 Time amount heating was carried out. After cooling, when reduced pressure distilling out of the solvent was carried out, the target compound 4 was obtained as a 4.4g solid. The compound 4 (5.1g; 0.028 mol) was dissolved in thionyl chloride 40mL, and it agitated at the room temperature for 4 hours (a compound 5

generates by this reaction). The superfluous thionyl chloride was dissolved after reduced pressure distilling out, residue was dissolved in chloroform 40mL, and chloroform solution 40mL of p-nitrophenol (12g; 0.086 mol) and triethylamine (8.7g; 0.086 mol) was dropped into it. The crystal was obtained, when the usual after treatment was performed after churning at the room temperature after dropping for 2 hours and the organic layer was distilled out. When this crystal was recrystallized with ethyl acetate / hexane mixed solvent, 7.2g of the target compounds 6 was obtained. They are 200mL(s) about a compound 6 (3.0g; 0.005 mol). 0.2N NaOH It adds to a solution and is 1.5 at 100 **. Time amount heating churning was carried out. The ether extracted repeatedly and the nitrophenol was removed, after acidifying with a hydrochloric acid. When water layers were collected and this was condensed by the evaporator, the white crystal deposited. Compound 7 (transition state analog)900mg made into the object by recrystallizing in ethanol It was obtained.

[0031]

[The synthetic example 2]

It combines with a carrier protein keyhole phosphorus ped hemocyanin (KLH) by making into hapten the compound 7 obtained in the example 1 of synthetic composition of hapten-KLH complex, and is the hapten for immunity. - KLH was compounded. It dissolved in the methylene chloride of 2mL(s), and hapten (7.5mg of compounds), N-hydroxy SAKUSHIMIDO (4mg), dicyclohexylcarbodiimide (7.4mg), and a pyrrolidino pyridine (0.4mg) were made to react at a room temperature for one day. KLH water solution after removing the generated urea object (6 mg/mL) It added calmly 1 mL and was made to react at a room temperature for 3 hours. Then, it dialyzed for two days with water, and hapten-KLH complex was compounded. By the completely same technique, the hapten-BSA complex for antibody titer measurement was compounded.

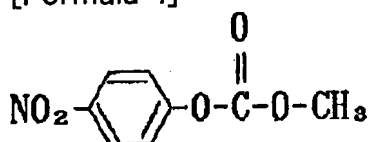
[0032]

[The synthetic example 3]

The substrate 1 of the following structure was compounded by the approach of a publication to synthetic J.Am.Chem.Soc. of a substrate, 109, and 2174-2176 (1987).

[0033]

[Formula 4]



[0034]

[Example]

(1-1) About the hapten-KLH complex compounded in the example 2 of production composition of an antibody enzyme, it is phosphoric acid buffer solution (10mM, pH 7.4, 0.9% NaCl content). It melted and considered as 40microg/mL solution. Thing 500 which mixed this with the equivalent Freund's complete adjuvant (it is used in a booster, mixing with Freund's incomplete adjuvant) muL Immunity was carried out to the Balb/C mouse. The booster was performed 4 times every three weeks. Then, after stopping immunity during February, every two weeks, 2 times immunity was carried out and the cell fusion of the spleen of an immunity mouse was carried out after the antibody titer check according to the conventional method [cell / (SP2) / ejection, this spleen cell, and / myeloma] using a polyethylene glycol. It screened by the ELISA method using the microtiter plate which fixed-hapten-BSA complex, and 93 sorts of antibody forming cells of hapten affinity were chosen. Abdominal cavity injection of this cell was carried out at the Balb/C mouse. The antibody content ascites produced by mouse intraperitoneal was collected, and IgG fractionation was collected with the ammonium-sulfate precipitation method. The protein A column (MAPS-2 kit; Bio-Rad make) refined the obtained IgG fractionation.

[0035] (1-2) The antibody with catalytic activity was selected by the following technique from antibodies with the hapten combining ability chosen in the assessment example 1-1 of the catalytic activity of an antibody enzyme. substrate 1 (0.12 mg/mL; 10 mM Tris-HCl buffer solution; pH8.5) 0.2mL of the synthetic example 3 a purification antibody (2mg/(ml)) -- 0.1mL absorption of the PARANITRO phenol which was mixed, and the substrate 1 disassembled and produced -- 400nm It pursued. From 93 sorts of antibodies, the antibody (antibody enzyme) with the capacity which decomposes a substrate was found 3 clone (the antibody enzyme four A1, 5H2, 1G2) (refer to drawing 3).

[0036] The purification IgG is made into 10 mg/mL (acetic-acid buffer solution; pH4.2) about the antibody enzyme four A1 whose catalytic activity was the highest, and it is 0.5mg to the 1mL. The pepsin (Sigma shrine make) was added and it was made to react at 37 degrees C for 20 hours. Gel filtration was carried out in Superdex-200 column (Pharmacia manufacture) after the reaction, and F(ab')₂ fraction was obtained.

[0037] (2) It is phosphoric acid buffer solution (10mM, pH 7.4, 0.9% NaCl content) about CRP (C-reactive protein) of production marketing of an anti-CRP antibody. It melts and is 400. It considered as mug/mL solution. Thing 500 which mixed this with the equivalent Freund's complete adjuvant (it is used in a booster, mixing with a physiological saline) muL Immunity was carried out to the Balb/C mouse. The booster was performed 5 times every two weeks. According to the conventional method [cell / (SP2) / ejection,

this spleen cell, and / myeloma] using a polyethylene glycol, the cell fusion of the spleen of an immunity mouse was carried out after the antibody titer check. Abdominal cavity injection of the antibody production syncytium screened and obtained by the ELISA method was carried out at the Balb/C mouse. The anti-CRP antibody content ascites produced by mouse intraperitoneal was collected, and IgG fractionation was collected with the ammonium-sulfate precipitation method. The protein A column (MAPS-2 kit; Bio-Rad make) refined the obtained IgG fractionation. Purification IgG is made into 10 mg/mL (acetic-acid buffer solution; pH4.2), and it is 0.5mg to the 1mL. The pepsin (Sigma shrine make) was added and it was made to react at 37 degrees C for 20 hours. Gel filtration was carried out in Superdex-200 column (Pharmacia manufacture) after the reaction, and F(ab')₂ fraction was obtained.

[0038] (3) 2-mercapto amine 0.05mL of 0.5M was added, and at 30 degrees C, it was made to react to 3 mg/mL solution (phosphate buffer; pH6) 0.45mL of four A1F(ab') antibody enzyme 2 fractionation produced by production (1-2) of a two-animal antibody for 90 minutes, and returned to it. Gel filtration is carried out with G-sephadex 25 column after a reaction, and it is moiety object Fab' of an antibody enzyme. It obtained. On the other hand, in 3 mg/mL solution (phosphate buffer; pH6) 0.45mL of F(ab')₂ fractionation of the anti-CRP antibody produced by (2), it is 5mM. Dithio SURAI toll 0.05mL was added, and at 30 degrees C, it was made to react for 30 minutes and returned. The thiol group which carried out 0.05mL addition of the after [a reaction] dithio screw nitro benzoic acid (50mM), and was returned while carrying out a reaction halt was masked. Gel filtration is carried out with this G-sephadex 25 column, and it is anti-CRP-Fab' of a moiety object. It obtained. In this way, moiety object Fab' of the obtained antibody enzyme and moiety object Fab' of an anti-CRP antibody It was made to meet by carrying out equivalent mixing and leaving it under a room temperature for 10 hours. Gel filtration of the united F(ab')₂ (two-animal antibody) was carried out, and Superdex-200 column (Pharmacia manufacture) refined it.

[0039] (4) As an example of the example comparison of a comparison, the antibody enzyme labelled antibody by the chemical bond was used. That is, antibody enzyme 4A1 monomer of (1-1) and the anti-CPP antibody monomer of (2) were combined by law two steps of glutaraldehydes, and anti-CRP-antibody enzyme complex was produced.

[0040] (5) it produced by production (2) of an anti-CRP fixed plate -- anti-- CRP-IgG(50microg/mL, PBS solution) 50microL It put into each well of 96 hole microtiter test plate (product made from Nunc), and sensitization was carried out at 4 degrees C overnight. Then, each well is washed by PBS and it is 300. muL 3%BSA The content PBS solution was put into each well, and the nonspecific adsorption site was blocked. In addition, in the sandwich

technique of this example, it combines with the antigenic determinant from which one antigen differs, and, originally the 1st solid-phase-ized antibody and the 2nd antibody used as the moiety object of a two-animal antibody use what has different antigenic specificity. However, since CRP was a pentamer, in this example, the same antibody as the anti-CRP antibody used for the two-animal antibody was solid-phase-ized. However, the solid-phase-ized anti-CRP antibody used intact IgG which has not carried out pepsin digestion.

[0041] (6) Dilute the quantum CRP of CRP with an PBS solution gradually, and it is every 50microL. It put into each well of the anti-CRP antibody fixation-ized plate produced by (5), and was made to react at 37 degrees C for 2 hours (the 1st immunoreaction). The PBS solution washed 3 times after that, and uncombined CRP was removed. Two-animal antibody produced by (3) to each well (10microg/mL) PBS solution 100microL to contain It was made to react for 60 minutes at 37 degrees C in addition (the 2nd immunoreaction). The PBS solution washed 3 times after that. Subsequently, it is the substrate 1 of the synthetic example 3 0.08 mg/mL It is the Tris-HCl buffer solution (pH8.5) to contain 200 muL In addition, it was left for 20 minutes at the room temperature. Then, it is 400nm of each well with a microplate reader (Corona Publishing make). The absorbance was measured and the calibration curve was created.

[0042] Anti-CRP-antibody enzyme complex produced instead of the two-animal antibody in the example of a comparison (4) (12microg/mL) The PBS solution to contain was used. The reaction is the same as the case of a two-animal antibody. As shown in drawing 4, the direction of an example (-O-) was about 10 time high sensitivity from the example of a comparison (---). This shows that the immunity analysis [high sensitivity it not only to have enzyme activity as an indicator reagent, but to consider as the two animal antibody combined with the moiety object of an anti-ligand antibody by making this into a moiety object] of an antibody enzyme is attained.

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- 2.*** shows the word which can not be translated.
- 3.In the drawings, any words are not translated.

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1] It is the explanation schematic diagram of the immunity analytical method of this invention.

[Drawing 2] It is drawing showing the synthetic path of the hapten (transition state analog) used in the example of this invention for antibody enzyme production.

[Drawing 3] It is drawing showing the catalytic activity ability of hapten affinity antibody 36 clone.

[Drawing 4] It is drawing showing the calibration curve which shows the result of an example and the example of a comparison.

[Description of Notations]

10 Solid Phase-ized Antibody,

12 Antigen (Ligand)

14 Two-Animal Antibody

14a The moiety object of an anti-ligand specific antibody

14b The moiety object of an enzyme-labelled antibody

[Translation done.]

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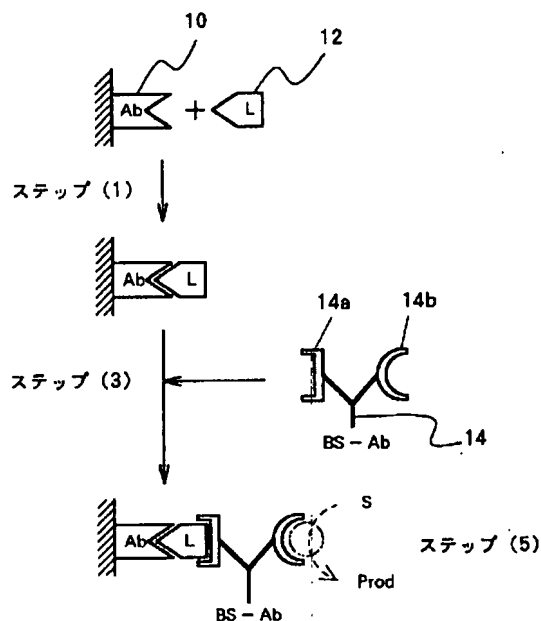
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(54)【発明の名称】 抗体酵素免疫分析法

(57)【要約】

【構成】 リガンドに対する抗体の半量体と、検出可能な信号を発生することのできる抗体酵素の半量体とからなる2頭抗体を、リガンドに接触させ、リガンドに結合した(又はしていない)2頭抗体の抗体酵素の活性を測定することを特徴とする抗体酵素免疫分析法。

【効果】 2頭抗体は通常の抗体と同様の2量体構造であり、化学的な修飾はされていないので抗体活性は損なわれていない。また、抗体酵素の酵素活性も、損なわれることがない。この2頭抗体以外には酵素等の添加を必要としないので、添加酵素の非特異吸着に伴うS/N比の低下等も生じない。抗リガンド抗体に対する抗体酵素の割合を最大比の1とすることになるから、高い検出感度が望める。従って高感度かつ再現性の良い分析ができる。



【特許請求の範囲】

【請求項1】 リガンドに対する抗体の半量体と、検出可能な信号を発生することのできる抗体酵素の半量体とからなる2頭抗体を、リガンドに接触させ、リガンドに結合した2頭抗体の抗体酵素の活性を測定することを特徴とする抗体酵素免疫分析法。

【請求項2】 リガンドに対する抗体の半量体と、検出可能な信号を発生することのできる抗体酵素の半量体とからなる2頭抗体を、リガンドに接触させ、リガンドに結合していない2頭抗体の抗体酵素活性を測定すること

【発明の詳細な説明】

【0001】

【産業上の利用分野】本発明は抗体酵素を用いる免疫分析法に関するものであり、詳しくは2頭抗体を用いた抗体酵素免疫分析法に関する。

【0002】

【発明の背景】血液や尿等の分析は、病態の診断や治療経過の判定に非常に有用であり、臨床検査の分野で重要な役割を果たしている。このような微量成分（リガンド）の分析方法として、該微量成分に対する抗体を用いて免疫学的に測定する方法が広く応用されている（例えば「免疫学イラストレイテッド」多田富雄訳、南江堂、1990年、p327-339参照）。免疫反応は抗原と抗体との間の特異的かつ高親和性の反応であり、抗体と抗原の平衡状態を標識抗原又は標識抗体とを用いて解析することにより測定対象の抗原を定量することができる。この標識として酵素を使用して酵素による化学増幅を利用したのが酵素免疫測定法（エンザイムイムノアッセイ；EIA）である。酵素免疫測定法は、簡便かつ高感度な分析方法として近年盛んに利用され、その詳細は例えば石川栄治、辻章夫ら編集、「酵素免疫測定法」（共立出版、1987年）に記載されている。

【0003】現在、抗原測定のために最もよく使用されている酵素免疫測定法はいわゆるサンドイッチ法である。サンドイッチ法は、2以上の抗原決定部位を持つ多価抗原の測定法であり、異なる抗原決定部位に対する2種類の抗体を用いる。代表的なサンドイッチEIA法は、例えば次のようなステップで行なうことができる。

1. 試料中の抗原を、固相化抗体（第1抗体）に結合させる（第1免疫反応）。
2. 未結合の抗原を除去する（洗浄）。
3. 酵素標識抗体（固相化抗体とは異なる第2抗体）を添加し、固相上の抗原に結合させる（第2免疫反応）。
4. 未結合の酵素標識抗体を除去する（洗浄）。
5. 酵素基質を加え、結合している酵素標識抗体量を測定する（酵素反応）。

このような方法では、第2抗体を酵素で標識し酵素標識抗体を作る必要があり、抗体と酵素を主に架橋試薬を用いて化学的に結合していた（例えば、石川栄治、河合

忠、宮井潔著「酵素免疫測定法」（医学書院、1987年）。

【0004】酵素免疫測定法では、使用する酵素標識抗体の性能が測定感度、再現性などに大きな影響を与える。しかし、抗体と酵素とを化学結合させて得られる酵素標識抗体は、この性能の点で問題がある。

【0005】架橋試薬として代表的なグルタルアルデヒドは、抗体、酵素のアミノ基と結合・架橋するが、その結合はランダムであり抗体、酵素のアミノ基に無差別に反応して抗体活性や酵素活性を損なう。また酵素のみが重合したり抗体のみが重合したりするため標識効率が低い。またランダムな反応の結果、ホモポリマーやヘテロポリマーが多くなり、これらポリマーは一旦形成されると、必要とする酵素標識抗体（酵素：抗体＝1：1の割合で結合している複合体が理想的である）との分離が困難であるため、EIA法の感度・再現性を低下させる原因となっていた。

【0006】過ヨウ素酸法は、酵素の糖鎖を酸化してアルデヒド基を形成させ、このアルデヒド基と抗体のアミノ基とを反応・結合させるものである。この方法では、酵素活性の低下は少ないものの、抗体のアミノ基に対する選択性はないので、抗体活性の低下は免れない。また糖鎖をもたない酵素には適用できない。

【0007】マレイミド法は、IgG抗体のヒンジ部にあるチオール基に、酵素に導入したマレイミド基を結合・架橋させる。この方法では、抗体活性部位から離れたヒンジ部分に選択的に酵素を結合できるので、抗体活性の低下は少ない。しかし、酵素のアミノ基に対する選択性はないから、酵素に2以上のアミノ基があれば酵素活性は多少とも低下する。また1分子の酵素に2分子以上の抗体が結合することになれば、抗体1分子当たりに対する標識酵素の濃度は低くなり、感度の上昇は望めない。

【0008】化学結合による酵素標識法は他にも種々あるが、得られる酵素標識抗体はいずれも上記と大同小異の欠点を有している。またこれら化学結合による酵素標識抗体は、抗体と酵素という分子量の大きい分子同士が結合したものであるため、マイクロタイタープレートやビーズ等の固相に対する非特異的吸着性が增大するという欠陥も生じやすい。そのため期待通りの感度が得られなくなったり、再現性が乏しくなるという問題もあった。

【0009】また、我々は、従来のような化学的に結合した酵素抗体複合体を用いる代りに、酵素を2頭抗体（BS抗体；Bi-specific antibody）に免疫学的に結合させて酵素免疫分析を行う方法も開発し、この性能も検討した（特願平3-113774）。この2頭抗体を用いる方法によって感度、再現性に著しい向上が見られた。しかしこの方法は、反応系に多量の酵素を添加しなければなら

ないため、添加酵素の非特異的吸着によりS/N比が低

下するという新たな問題が生じてきた。

【0010】

【発明の目的】本発明は、以上のような事情に鑑みなされたものであり、抗体活性の低下が少なく、高感度かつ再現性の良い分析が、S/N比の低下を伴わずに行なうことが出来る免疫測定法を提供することを目的とする。

【0011】

【発明の構成】このような本発明の目的は、リガンドに対する抗体の半量体と、検出可能な信号を発生することのできる抗体酵素の半量体とからなる2頭抗体を、リガンドに接触させ、リガンドに結合した（又はしていない）2頭抗体の抗体酵素の活性を測定することを特徴とする抗体酵素免疫分析法により達成された。

【0012】

【作用】本発明では、従来のような化学的に結合した酵素抗体複合体を用いる代りに、抗体酵素の2頭抗体（B S抗体；Bi-specific antibody）を用いて免疫分析を行う。これは、検出可能なシグナルを発生することのできる抗体酵素の半量体と、被検物（リガンド）に対する抗原特異性を有する特異抗体の半量体とからなる2頭抗体を用いることにより、問題となる感度の低下が見られなくなるという発明者による知見に基づくものである。

【0013】2頭抗体は通常の抗体と同様の2量体構造であり、化学的な修飾はされていないので抗体活性は損なわれていない。また、抗体酵素の酵素活性も、損なわれることがない。また、この2頭抗体以外には酵素等の添加を必要としないので、添加酵素の非特異吸着に伴うS/N比の低下等も生じない。

【0014】

【発明の構成の詳細な説明】

抗体酵素

本発明における抗体酵素とは、その特異的結合対を別の生成物に換える能力を有する抗体のいう。言い換えれば、酵素活性（または触媒活性）を保持する抗体のことである。抗体酵素(Antibody Enzyme)は、また、触媒抗体(catalytic Antibody)、Abzyme等と呼ばれるよばれることもある。

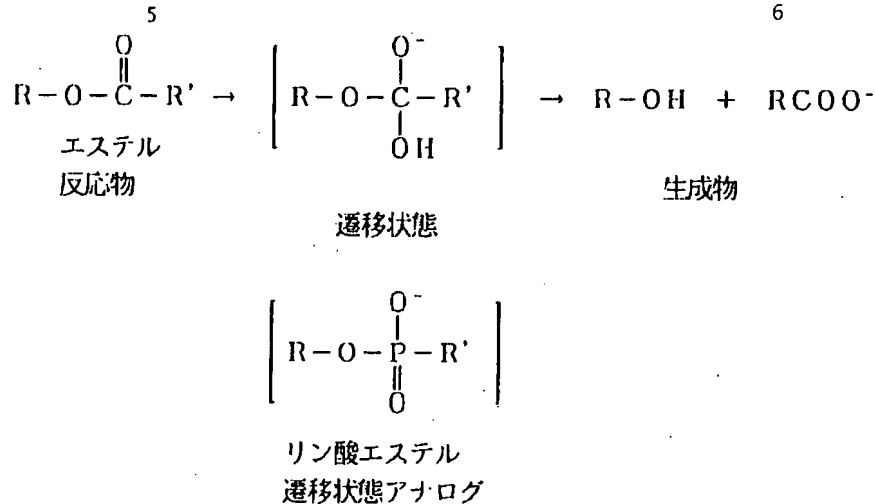
【0015】一般に、抗体は特定の分子（抗原）を識別して特異的に結合する蛋白質として特徴づけられる。一方、同じ蛋白質である酵素は特定の分子（酵素基質）に

特異的に結合するだけでなく、その分子の化学反応を触媒する機能を有する。すなわち、酵素も抗体も特異的な物質と結合対をつくることのできる生体内蛋白質ではあるが、抗体は、化学反応を触媒する能力がないという点で、酵素と区別することができる。しかし、抗体は原理的のほとんど全ての分子に対して特異的なものを得ることが可能であることから、抗体と酵素とが共有する特性、すなわち特異的に結合するという性格を基礎にして、化学反応を触媒するような抗体を得ることができないかとの試みがなされた。ただ初期の研究では、基質に対する抗体を作製するものであったため、触媒効果を持つ抗体を見つけるには至らなかった(Biochemistry, 5, 2836(1966)、FEBS Letter, 100, 137(1979))。これに対して、P.G.SchulzらやR.A.Lernerらは、“酵素の活性中心は、反応の遷移状態と相補的な構造を持つ”というL.Paulingの考えに基づき、反応の遷移状態のアナログに対する抗体を作製し、この抗体が触媒活性を持つことを発見した(Science, 234, 1570(1986)、Science, 234, 1566(1986))。その後、多数の研究者によりこのような触媒活性を持つ抗体が作製され、抗体酵素(触媒抗体, Abzyme)という考えが広く認められるに至っている。

【0016】遷移状態アナログとは、ある酵素反応において遷移状態にある物質(中間体)と形も電荷もよく似た安定な類似体であり、このような遷移状態アナログは、抗原として働いて抗体を誘導し、又できた抗体は、反応過程の遷移状態にある物質に結合し、これを安定化し、さらに触媒として機能する。例えばカルボン酸エステルの加水分解反応では、反応物であるエステルは一般に電荷をもたない平面上の分子構造をとる。加水分解は水分子の攻撃により始まり、電荷をもった四面体型中間体(遷移状態)を経たのち、速やかにカルボン酸とアルコールに分解するという経過をたどる。この中間体では、原子間の結合方向も変わるし、原子間距離も約1.2倍ほどに伸びる。このような不安定な特徴からこれを単離することはできず、その抗体を得ることはできない(下記化1参照)。

【0017】

【化1】



【0018】しかし、この四面体構造の中央の炭素原子(C)をリン原子(P)に置き換えると、よく似た立体配置をとるリン酸エステルと呼ばれる安定な化合物となる。しかもリン-酸素間の結合距離は、通常の炭素-酸素間の結合よりも約20%長く、実際の遷移状態の結合距離に近い。実際このような性質を備えたリン酸エステルは、ある種の加水分解酵素を阻害することが知られている。このような遷移状態アナログを抗原として免疫することにより、この酵素活性をもった抗体、すなわち抗体酵素を得ることができる。

【0019】本発明における2頭抗体の半量体の一方として使用する抗体酵素とは、このような酵素活性を有する抗体をいう。本発明では抗体酵素を、従来の標識酵素の代わりとして用いる。従って、ここで使用する抗体酵素とは、基質から、検出可能な信号を発生することのできる生成物又は分解物を生じることができるものである。例えば、前記式1中のエステル分解により生じる生成物が色素であるような物質を基質とするものがある。なお、抗体酵素としては、遷移状態アナログをそのまま免疫して得られたポリクローナルな抗体をそのまま用いるよりも、モノクローナル抗体を作製して、高い触媒活性を有するものを選択して、これを本発明の2頭抗体の材料とするのが好ましい。

【0020】2頭抗体

免疫グロブリン(抗体)は1種類のものではなく、その化学構造から幾つかのクラスに分類されるが、その基本構造はIgGに見られる構造である。すなわち、分子量約25,000のL鎖と分子量約50,000のH鎖とがS-S結合(ジスルフィド結合)して1つの単位(半量体)となり、これら等価な半量体がさらにH鎖のS-S結合で2量体化して1つのIgG分子を形成する。抗原結合部位(Fab部位)は各半量体にそれぞれ存在する。つまり、IgGは抗原特異性は1つであるが2つの抗原分子に結合する能力(同種2価)を有している。この同種2価のIgGを化学的に処理することにより異なる2つの抗原

特異性を有する抗体(異種2価のいわゆる2頭抗体)を作ろうとする概念自体は既に存在し、種々の方法が開発されている。本発明で使用する2頭抗体はこれら公知技術により作ることができ、検出可能なシグナルを発生することのできる抗体酵素の半量体と、被検物(リガンド)に対する特異抗体の半量体とから構成される。

【0021】例えば、Nisonoffら方法に従い、2種のIgGをペプシン消化してFc部分を除去した後、それぞれ還元して半量体のFab'を得、これを再酸化することにより2頭抗体を作成できる(Arch. Biochem. Biophys., 90, 460-462, (1961))。この方法では、相異なる抗体の半量体Fab'が結合した異種2価抗体の他に、同じ抗原特異性の半量体Fab'が結合(自己会合)した同種2価抗体も形成される。従ってこの場合には、同種2価抗体から異種2価抗体を分離・精製する。或いは、Brennanらの方法に従い、半量体Fab'の一方をSH基をニトロ安息香酸で一時的に保護してもよい(Science, 229, 81, (1985))。これにより2種のモノクローナル抗体から異種2価の2頭抗体を選択的に作成できる。Brennanらの手法をさらに簡便化した奥村らの方法に従ってもよい(特開平2-76899)。

【0022】なお上記の2頭抗体では、いずれもFc部分を除去したF(ab')₂を用いているが、Fc部分を除去しないで還元して半量体を得て、これを他の半量体と酸化・結合させてもよい。しかし、Fc部分は固相に非特異的吸着しやすい。従って、高感度測定のためには、IgGをペプシン消化してFc部分を除去し、さらに還元して得られるFab'を2頭抗体の材料とするのが望ましい。

【0023】また、2頭抗体は、2種類のハイブリドーマを融合し、ヘテロハイブリドーマを作製することによっても作製することができる。また、ハイブリドーマと、脾臓細胞を融合することによっても作製することができる。モノクローナル抗体の作成方法、その精製方法、F(ab')₂断片の取得方法は種々の成書に記載されて

いる方法により得ることができる（例えば、富山朔二ら編、「単クローン抗体実験マニュアル」講談社刊、1988年）。

【0024】分析方法

本発明による抗体酵素免疫分析法は、具体的には以下のように行うことができる。（図1参照）。

- 1) 試料中の抗原（リガンド）12を固相化抗体10に結合させる（第1免疫反応）。
- 2) 未結合の抗原12を除去する（洗浄）。
- 3) リガンド特異抗体の半量体14aと酵素抗体の半量体14bからなる2頭抗体（BS-Ab）14を添加し、固相化抗原10に結合している抗原12に結合させる（第2免疫反応）。
- 4) 抗原12に未結合の2頭抗体を除去する（洗浄）。
- 5) 酵素基質（S）を加え、生成物（Prod）を検出することにより結合した2頭抗体量を測定する（酵素反応）。

【0025】以上は、サンドイッチ法に本発明を適用し固相に結合した2頭抗体の抗体酵素活性を測定するよう*

*にしたものであるが、固相に結合しなかった遊離の2頭抗体に結合した抗体酵素の活性を測定するようによい。また本発明の実施方法がこのようなサンドイッチ法に限定されるわけではない。例えば、一定量の固相抗原と試料中の抗原（リガンド）とを一定量の2頭抗体に対して競合させ、固相に結合した2頭抗体の量を抗体酵素活性測定により求めるようによい。また固相抗原に予め2頭抗体を結合させておき、後から添加した試料中の抗原（リガンド）との競合により減少した固相上の2頭抗体の量を測定するようによい（いわゆる置換法）。

【0026】

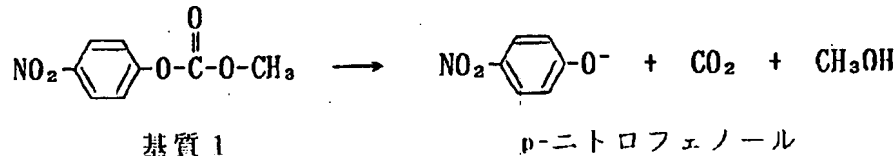
【合成例1】

抗体酵素用ハブテンの合成

下記反応式により基質1からp-ニトロフェノール（黄色：400nmに吸収）を生成する反応を想定し、これを触媒する酵素抗体の作製を行なった。

【0027】

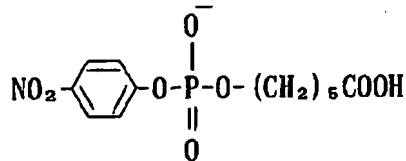
【化2】



【0028】まず上記反応の遷移状態アナログ（ハブテン）として下記構造式の化合物を合成した。

【0029】

【化3】



【0030】その反応合成経路の概略を図2に示す。まず、図2の化合物1（17g; 0.1モル）と化合物2（20g; 0.1モル）を混合し、160℃にて7時間加熱した。冷却後、蒸留により低沸点部分を溜去すると目的とする化合物3が13g無色油として得られた。化合物3（6.5g; 0.026モル）を10mLのエタノールに溶解し、12N塩酸を80mL加えた。混合物を160℃にて2.5時間加熱した。冷却後、溶媒を減圧溜去すると目的とする化合物4が4.4g固形物として得られた。化合物4（5.1g; 0.028モル）を塩化チオニル40mLに溶解させ、4時間室温にて攪拌した（この反応により化合物5が生成する）。過剰の塩化チオニルを減圧溜去後、残渣をクロロホルム40mLに溶解し、その中にp-ニトロフェノール（12g; 0.086モル）とトリエチルアミン（8.7g; 0.086モル）のクロロホルム溶液40mLを滴下した。滴下後、2時間室温にて攪拌

後、通常の後処理を行い、有機層を溜去すると結晶が得られた。この結晶を酢酸エチル/ヘキサン混合溶媒にて再結晶すると、目的とする化合物6が7.2g得られた。化合物6（3.0g; 0.005モル）を200mLの0.2N NaOH溶液に加えて、100℃にて1.5時間加熱攪拌した。塩酸にて酸性化した後、エーテルにて繰り返し抽出してニトロフェノールを除去した。水層を集め、これをエバポレーターで濃縮すると、白色結晶が析出した。エタノールにて再結晶することにより、目的とする化合物7（遷移状態アナログ）900mgが得られた。

【0031】

【合成例2】

ハブテン-KLH複合体の合成

合成例1で得た化合物7をハブテンとして、担体蛋白質キーホール・リンベッド・ヘモシアニン（KLH）に結合し免疫用のハブテン-KLHを合成した。ハブテン（化合物7：5mg）、N-ヒドロキシサクシミド（4mg）、ジシクロヘキシルカルボジイミド（7.4mg）、ピロリチノビリジン（0.4mg）を2mLの塩化メチレンに溶解し、室温で1日反応させた。生成した尿素体を除去後、KLH水溶液（6mg/mL）を1mL静かに添加し、室温で3時間反応させた。その後、水で2日間透析し、ハブテン-KLH複合体を合成した。全く同様な手法により、抗体力価測定用のハブテン-BSA複合体を合成した。

【0032】

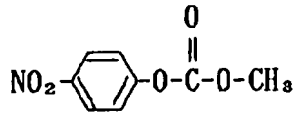
【合成例3】

基質の合成

J. Am. Chem. Soc., 109, 2174-2176 (1987) に記載の方法により、下記構造の基質1を合成した。

【0033】

【化4】



【0034】

【実施例】

(1-1) 抗体酵素の作製

合成例2で合成したハプテン-KLH複合体を、磷酸緩衝溶液(10mM, pH7.4, 0.9% NaCl含有)に溶かし、40μg/mL溶液とした。これを等量のプロイント完全アジュバントと混合したもの(追加免疫では、プロイントの不完全アジュバントと混合して使用)500μLを、Balb/Cマウスに免疫した。追加免疫は、3週間ごとに4回行った。その後、2月間免疫を中止した後、2週間ごとに2回免疫をし抗体力価確認後、免疫マウスの脾臓を取り出し、この脾臓細胞とミエローマ細胞(SP2)とをポリエチレングリコールを用いる常法に従って細胞融合させた。ハプテン-BSA複合体を固定したマイクロタイタープレートを使用してELISA法でスクリーニングし、ハプテン結合性の抗体産生細胞を93種選択した。この細胞をBalb/Cマウスに腹腔注射した。マウス腹腔内で生産された抗体含有腹水を集め、硫酸アンモニウム沈澱法によりIgG分画を回収した。得られたIgG分画は、プロテインAカラム(MAPS-2キット; バイオラッド社製)により精製した。

【0035】(1-2) 抗体酵素の触媒能力の評価

実施例1-1で選んだハプテン結合能力のある抗体の中から、触媒能力の有る抗体を、次のような手法で選びだした。合成例3の基質1(0.12mg/mL; 10mM Tris-HCl 緩衝液; pH8.5)0.2mLと、精製抗体(2mg/mL)0.1mLを混合し、基質1が分解して生じたパラニトロフェノールの吸収を400nmで追跡した。93種の抗体より、基質を分解する能力のある抗体(抗体酵素)が、3クローン(抗体酵素4A1, 5H2, 1G2)見つかった(図3参照)。

【0036】最も触媒能力が高かった抗体酵素4A1について、その精製IgGを10mg/mL(酢酸緩衝液; pH4.2)にして、その1mLに0.5mgのペプシン(Sigma社製)を添加し、37°Cで20時間反応させた。反応後、Superdex-200カラム(ファルマシア社製)でゲル濾過し、F(ab')₂画分を得た。

【0037】(2) 抗CRP抗体の作製

市販のCRP(C反応性蛋白)を磷酸緩衝溶液(10mM, pH7.4, 0.9% NaCl含有)に溶かし、400μg/mL溶液とした。これを等量のプロイント完全アジュバントと混合したもの(追加免疫では、生理食塩水と混合して使用)500μLを、Balb/Cマウスに免疫した。追加免疫は、2週間ごとに5回行った。抗体力価確認後、免疫マウスの脾臓を取り出し、この脾臓細胞とミエローマ細胞(SP2)とをポリエチレングリコールを用いる常法に従って細胞融合させた。ELISA法でスクリーニングして得られた抗体産生融合細胞をBalb/Cマウスに腹腔注射した。マウス腹腔内で生産された抗CRP抗体含有腹水を集め、硫酸アンモニウム沈澱法によりIgG分画を回収した。得られたIgG分画は、プロテインAカラム(MAPS-2キット; バイオラッド社製)により精製した。精製IgGを10mg/mL(酢酸緩衝液; pH4.2)にして、その1mLに0.5mgのペプシン(Sigma社製)を添加し、37°Cで20時間反応させた。反応後、Superdex-200カラム(ファルマシア社製)でゲル濾過し、F(ab')₂画分を得た。

【0038】(3) 2頭抗体の作製

(1-2)で作製した抗体酵素4A1のF(ab')₂分画の3mg/mL溶液(磷酸緩衝液; pH6)0.45mLに、0.5Mの2-メルカプトアミン0.05mLを添加し、30°Cで90分反応させて還元した。反応後、セファデックスG-25カラムによりゲル濾過して、抗体酵素の半量体F(ab')₂を得た。一方、(2)で作製した抗CRP抗体のF(ab')₂分画の3mg/mL溶液(磷酸緩衝液; pH6)0.45mLには、5mMのジチオスライトール0.05mLを添加し、30°Cで30分反応させて還元した。反応後ジチオビスニトロ安息香酸(50mM)を0.05mL添加して反応停止すると共に還元されたチオール基をマスキングした。このセファデックスG-25カラムによりゲル濾過して、半量体の抗CRP-F(ab')₂を得た。こうして得られた抗体酵素の半量体F(ab')₂と、抗CRP抗体の半量体F(ab')₂とを、等量混合し室温下10時間放置することにより、会合させた。結合したF(ab')₂(2頭抗体)はSuperdex-200カラム(ファルマシア社製)でゲル濾過して精製した。

【0039】(4) 比較例

比較例として、化学結合による抗体酵素標識抗体を用いた。すなわち、(1-1)の抗体酵素4A1単量体と、(2)の抗CRP抗体単量体とを、グルタルアルデヒド2段階法で結合させ、抗CRP-抗体酵素複合体を作製した。

【0040】(5) 抗CRP固定化プレートの作製

(2)で作製した抗CRP-IgG(50μg/mL, PBS溶液)50μLを、96穴マイクロタイターテストプレート(Nunc社製)の各ウェルに入れ、4°Cで一晩感作した。その後、各ウェルをPBSで洗浄し、300μLの3%BSA含有PBS溶液を各ウェルに入れて、非特異的吸着部位をブロックした。なお本実施例のサンドイッチ法では、固相化する第1抗体と2頭抗体の半量体とされた第

2抗体とは1つの抗原の異なる抗原決定基に結合するものであり、本来は異なる抗原特異性を有するものを使用する。しかし、CRPは5量体であるので、本実施例では、2頭抗体に使用した抗CRP抗体と同じ抗体を固相化した。ただし固相化した抗CRP抗体はペプシン消化していないインタクトなIgGを用いた。

【0041】(6) CRPの定量

CRPをPBS溶液で段階的に希釈し、各50μLを

(5)で作製した抗CRP抗体固定化プレートの各ウェルに入れ、37℃で2時間反応させた(第1免疫反応)。その後PBS溶液で3回洗浄して、未結合のCRPを除去した。各ウェルに(3)で作製した2頭抗体(10μg/mL)を含有するPBS溶液100μLを加え、37℃で60分間反応させた(第2免疫反応)。その後PBS溶液で3回洗浄した。次いで、合成例3の基質1を0.08mg/mL含有するTris-HCl緩衝液(pH8.5)を200μL加え、室温で20分間放置した。その後、マイクロプレートリーダー(コロナ社製)で各ウェルの400nm吸光度を測定して、検量線を作成した。

【0042】比較例では、2頭抗体の代わりに、(4) 20で作製した抗CRP-抗体酵素複合体(12μg/mL)を含有するPBS溶液を使用した。反応は、2頭抗体の場合*

*と同じである。図4に示すように、実施例(—○—)の方が比較例(—●—)よりも約10倍高感度であった。このことは、抗体酵素は標識試薬として酵素活性を有するだけでなく、これを半量体として、抗リガンド抗体の半量体と組み合わせた2頭抗体とした方が、より高感度な免疫分析が可能となることを示している。

【図面の簡単な説明】

【図1】本発明の免疫分析方法の説明概略図である。

【図2】本発明の実施例において抗体酵素作製のために使用したハプテン(遷移状態アナログ)の合成経路を示す図である。

【図3】ハプテン結合性抗体36クローンの触媒活性を示す図である。

【図4】実施例及び比較例の結果を示す検量線を示す図である。

【符号の説明】

10 固相化抗体、

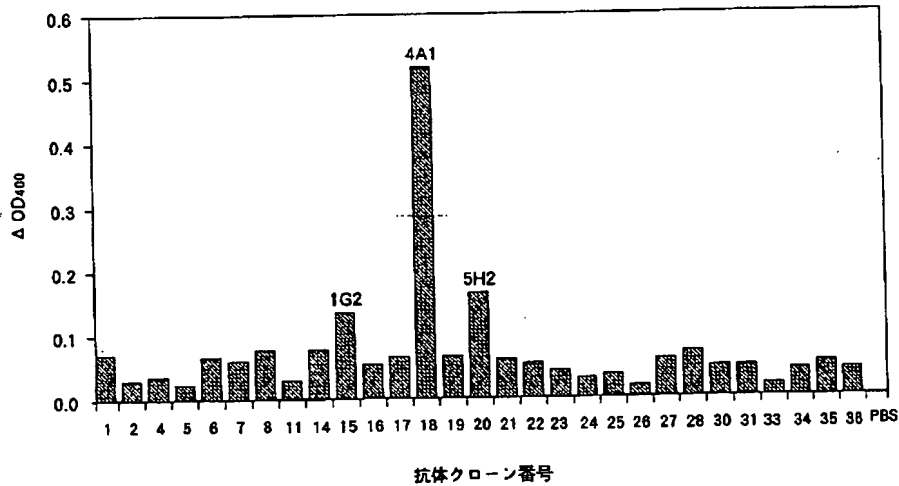
12 抗原(リガンド)

14 2頭抗体

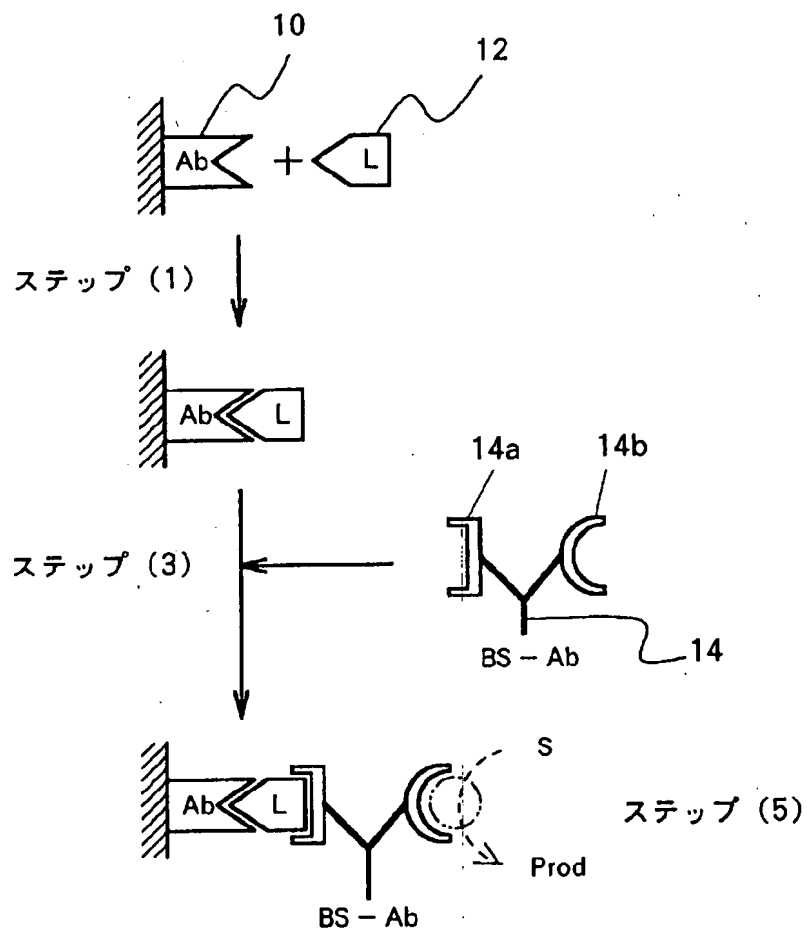
14a 抗リガンド特異抗体の半量体

14b 酵素抗体の半量体

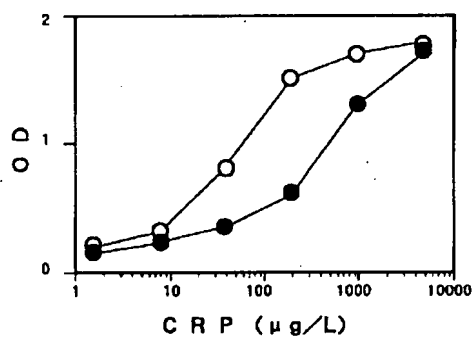
【図3】



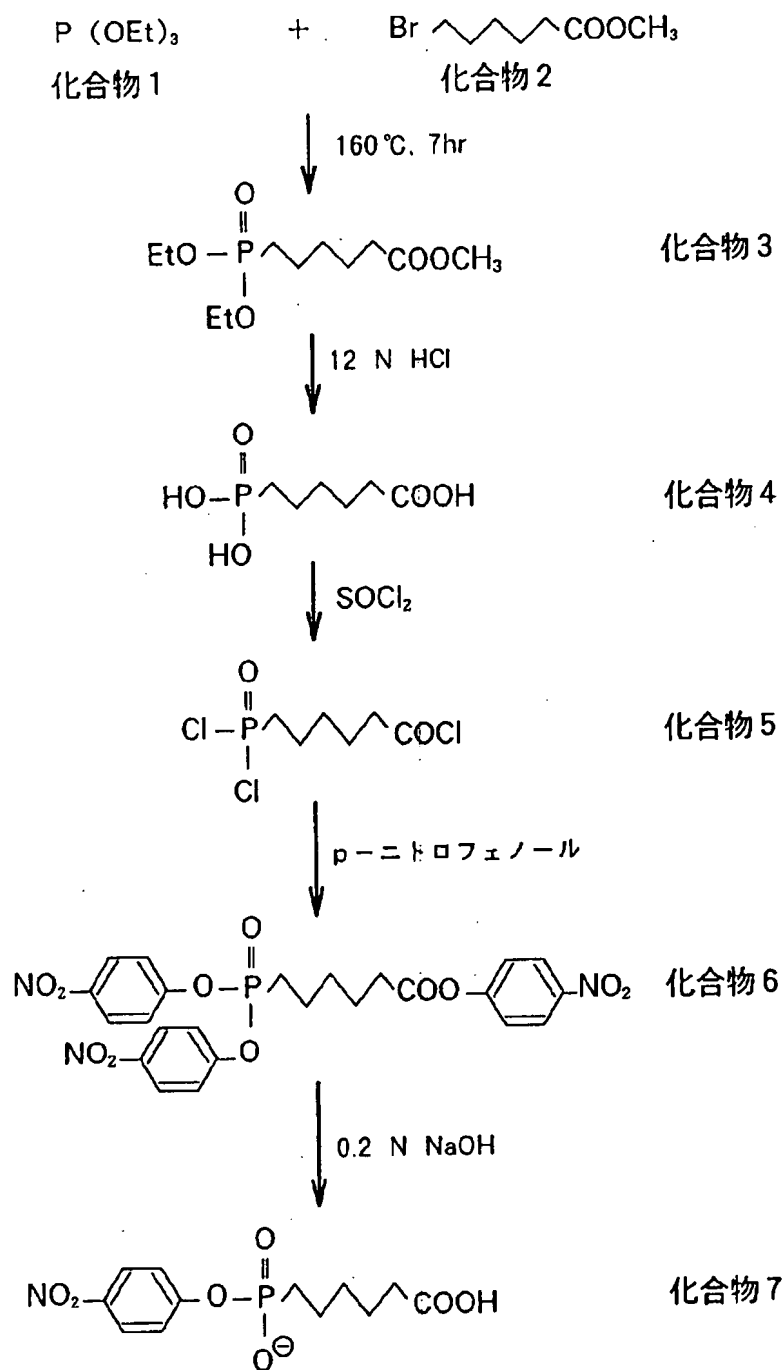
【図1】



【図4】



【図2】



フロントページの続き

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